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Gonad Response to γ-Aminobutyric Acid in the Sea Urchin

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ABSTRACT—In the sea urchin, *Strongylocentrotus intermedius*, gonad response to γ-aminobutyric acid (GABA) was examined by indirect measurements of gonad contraction. (1) Mechanical response was noted to increase with gonad growth. Young growing gonads showed small response but for maturing, ripe and post-spawning specimens, the response was large. (2) Two different GABA responses on the part of maturing, ripe and post-spawning gonads, and young and growing gonads were thus in evidence. In the former, there were both phasic and rhythmical contractions but in the latter, only phasic contraction. (3) GABA response was also noted to vary according to sex. In the female, the appearance of rhythm was high, but low in the male. (4) The duration of GABA response was about 20 min in maturing and ripe gonads. (5) The threshold concentration of GABA response was 0.01 mM. The 50% effective and maximal doses were 0.1 mM and 10 mM, respectively. (6) The sites of action of GABA injected at a concentration of 10 mM were examined. GABA acted directly on the gonads. The generator inducing phasic and rhythmical contraction is discussed.

INTRODUCTION

Several studies for obtaining gametes from sea urchins have already been performed, such as those involving the use of mechanical damage [1], potassium [1–3] and calcium [1] ions, electrical shock [4–7], acetylcholine [8], and the radial nerve factor [9, 10]. However, no report has so far appeared on a physiological triggering mechanism for gamete shedding in sea urchins.

The injection of γ-aminobutyric acid (GABA) into sea urchins was recently found to induce gamete release [11]. The threshold concentration of GABA for spawning was noted to be about 0.01 mM and the optimal for 100% spawning, to exceed 0.1 mM. The initiation time of release following the injection was about 12 sec. Thus GABA spawning is a rapid phenomenon. In the present research, this matter was examined in greater detail by measuring the mechanical response of gonads toward GABA. Portions of this work recently appeared in abstract form [12].

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MATERIALS AND METHODS

Animals

Sea urchins (*Strongylocentrotus intermedius*) were collected from the coast of Rishiri Island off northern Hokkaido throughout the year. The animals were kept at our laboratory in an aquarium provided with running seawater until use.

Seawater

Modified van't Hoff seawater (ASW) (462 mM NaCl; 9 mM KCl; 9 mM $CaCl_2$; 36 mM $MgCl_2$; 17 mM $MgSO_4$; 20 mM Tris-HCl, pH 8.2) was used as the basal incubation medium. K^+-rich ASW contained KCl at the concentration of 500 mM. NaCl was removed. The composition of other ions was the same as ASW. GABA ASW was made by adding γ-aminobutytic acid (Nakarai Chemicals Ltd.) at various concentrations to ASW.

Apparatus

Okada *et al.* [13] reported the central part of gonads at the time of spawning to increase in height and termed this height change as potential

difference (PD). The present study also uses this term as well as some of their procedures.

The sea urchins were first fixed with large forceps. On the oral side of each specimen, a hole 3 cm in diameter was opened by cutting the shell test with solid scissors. A small hole 5 mm in diameter on a portion of the remaining test was made by a dental drill. The animal was fixed by placing one foot of the forceps into a small hole and pinching the test (Fig. 1i).

The PD of the gonads was measured by a strain guage (Fig. 1c; SB–1TH, Nihon Koden). The strain guage was horizontally set and connected to a straw. At a certain point on the horizontally set straw, it was then placed perpendicularly and cut vertically in half with its top made rectangular. It was placed perpendicularly on the central part of the gonad (Fig. 1j). The PD measurement was amplified (Fig. 1b; RP–3, Nihon Koden) and recorded on an ink-writing oscillograph (Fig. 1d; Nihon Koden).

Solution exchange was carried out by withdrawing the old solution through the straw inserted in the body cavity (Fig. 1h) and then introducing the new solution through a 10 ml pipette (Fig. 1g).

Recording

The recording was performed at room temperature which varied from 18 to 25°C. The Aristotle's lantern and oesophagus were removed from the sea urchin. In experiments on the action site of GABA, the oral and aboral intestines were also removed by forceps. The animal was fixed and immersed up to the equator into filtered natural sea water in a large beaker (Fig. 1i and e). Soon after the animal had been fixed, its body cavity was filled with coelomic fluid or ASW by pipette. The straw was placed on the gonad and the siphon in the central portion of the body cavity. In that state, the sea urchin was left for 30 min. When the gonad was immersed in ASW, exchange of ASW was carried out two or three times during this period. After the 30 min, the gonads were treated with GABA for 10 min (Fig. 2). The PD induced by GABA was designated as PD_G and the GABA was removed. The gonad

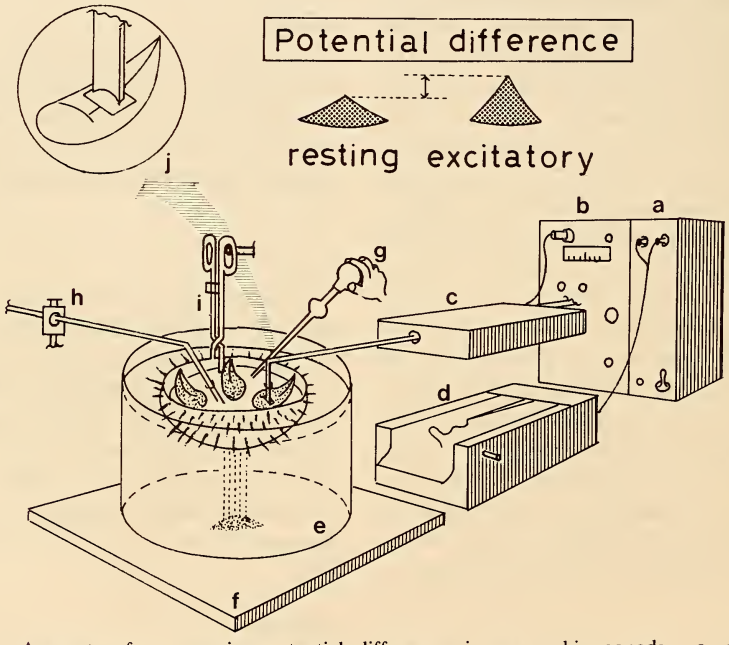

Fig. 1. Apparatus for measuring potential differences in sea urchin gonads. a, main amplifier; b, carrier amplifier; c, strain guage; d, ink-writing oscillograph; e, beaker; f, lab jacks; g, pipette; h, siphon; i, forceps; j, magnification of straw cut vertically in half, with the top made rectangular.

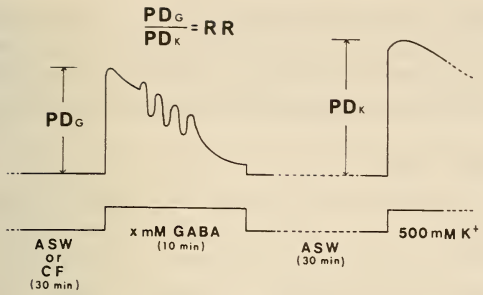

FIG. 2. Method for recording. ASW, artificial sea water; CF, coelomic fluid; PD_G, potential difference induced by GABA; PD_K, potential difference by 500 mM K^+; RR, relative response.

was washed two or three times in ASW and then left for 30 min. This was followed by treatment with K^+-rich ASW. The resulting PD was designated as PD_K. The relative value of PD_G against PD_K was designated as the relative response, RR.

Histology

To observe gonoduct and gonopore structures, the sea urchin was first placed in decalcified solution for about one week to adequately soften the test. The solution contained 5 ml nitric acid, 10 ml formalin and 85 ml distilled water. The decalcified sea urchin was cut into small pieces, which were then washed in running water. The gonaoduct and gonopore segments were embedded in paraffin according to the usual method and sectioned and stained with hematoxylin-eosin.

The silver-staining method for nerve cells was carried out as follows: small pieces of gonad were fixed with 10% formalin for 6 hr followed by washing in running water for 4 hr. Five drops of ammonia water were added to the solution containing the pieces. Twenty-four hours later, they were transferred to a 1.5% silver nitrate solution at 38°C and left for 5 days. The pieces were then placed in a reducing solution consisting of 100 ml distilled water, 15 ml formalin and 1 g hydroquinone. This was followed by embedding in paraffin by the usual method. After sectioning and removal of the paraffin, they were dehydrated and observed without stain.

RESULTS

Mechanical response

Contraction mode and sex differences Mechanical response to GABA was generally of two types. The response in young and growing gonads showed smooth phasic contraction (Fig. 3a). That in maturing, ripe and post-spawning gonads indicated oscillating rhythmical contraction superimposed on phasic contraction (Fig. 3b). Response to GABA ceased at about 20 min (Fig. 4). The time from the start of contraction to its peak was approximately 40 sec. Both small and large oscillations in rhythmical contraction were noted. On some occasions, rhythm could be observed to a small degree near the peak of the phasic contraction (Fig. 3a). Usually, large

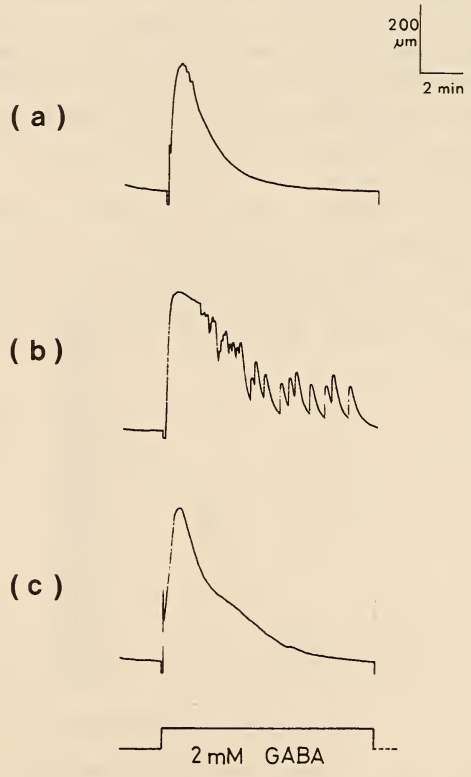

FIG. 3. Response to 2 mM GABA. a, a phasic contraction in growing ovary; b, a rhythmical contraction in addition to phasic contraction in the descending phase of a mature ovary; c, a phasic contraction in a testis.

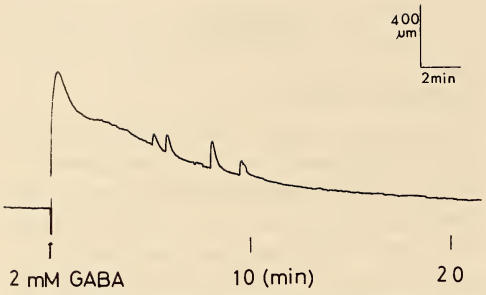

Fig. 4. Mechanical response induced by 2 mM GABA, in a mature ovary. Gonad response ceased at 20 min.

rhythm could be easily detected in females with mature ripe gonads. Males displayed only phasic contraction in response to GABA (Fig. 3c). Thus, response to GABA varies according to sex.

Seasonal variation Seasonal variation of GABA response was observed. RRs in January (1 mM GABA), May (2 mM) and July (2 mM) were 0.89±0.08 (mean±SEM), 0.60±0.11 and 1.03±0.08, respectively. RR in April through June was usually low. In Rishiri Island, the average weight of one lobule from a gonad was 0.86 g in May and 1.39 in July in sea urchins 4–5 cm in diameter. Since one lobule in post-spawning and young gonads is usually 0.32 g, the gonads in May were apparently in the growing stage, thus accounting for the response in gametogenic gonads. RR in July was significantly higher than in May ($P<0.01$). This high RR exceeding 1.0 may possibly have been due in part to the abundant release of gametes during the time when the gonads were treated with GABA.

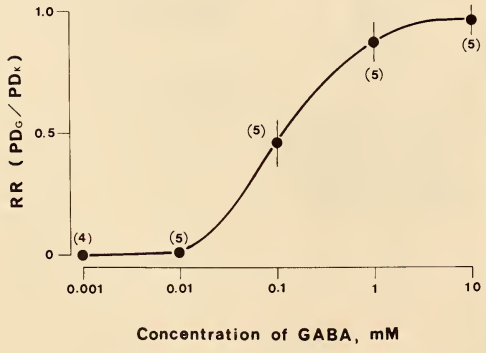

Fig. 5. Dose-response curve of gonad contraction by GABA. Abscissa, concentration of GABA (mM); ordinate, relative response (RR). Points and vertical bars are means±SEM. The number of preparations examined is shown in parentheses. 10 mM GABA corresponds to 0.96 RR.

Dose-response

Dose-response in the present study is represented as the response of PD toward GABA relative to that toward 500 mM K^+. The experiment was performed in February, when the gonads appeared to be in the post-spawning stage and the average weight of one lobule was 0.32 g for sea urchins 4–5 cm in diameter. PD peaks were measured as RR. In Figure 5, the threshold concentration is 0.01 mM. The 50% effective dose appeared near 0.1 mM and maximal one, 10 mM. The RR in 10 mM GABA was 0.96.

Site(s) of action

Aboral nerve system The aboral nerve sys-

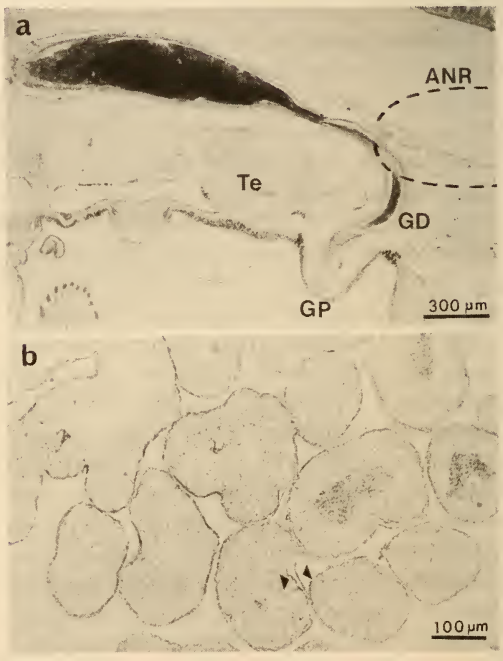

Fig. 6. (a) Histology of male sea urchin reproductive system. The test is decalcificated. ANR, aboral nerve ring; GD, gonoduct; GP, gonopore; Te, test. Dotted line indicates a supposed place of aboral nerve ring. (b) Testis stained with silver. Both layers in the gonad wall are stained (two arrow heads).

tem appears to be composed of nerve cells and fibers in the nerve ring surrounding the anus, those in the gonoduct and those in the gonad wall. The gonad wall in this animal is composed of three layers, outer coelomic epithelium (visceral peritoneum), middle connective tissue with muscle cells and nerve cells, and an inner germinal layer [14]. Silver-staining indicated two layers of nerve cells, one just beneath the outer coelomic epithelium and the other in the middle connective tissue (Fig. 6b). Some of the nerve cells in the gonad are also possibly associated with those in the gonoduct. As can be seen from Figure 6a, the gonoduct passes through the test and opens to the outside of the gonopore. At the point where the gonoduct enters the test, the nerve process in the gonoduct divides into two branches. By conjugating circularly with branches originating from each of the five lobules in the gonad, the aboral nerve ring is formed and surrounds the anus.

Site(s) of action The experiment was performed in March, at the time gonads were in the resting stage. The RR for 10 mM GABA was 0.8 (Fig. 7, control in b). To study the site(s) of action of injected GABA, three different experiments were carried out. The first experiment was conducted to determine if a supposed action site was the aboral nerve ring. One lobule was separated from the other four in a gonad by cutting nervous branches at both sides of the gonoduct with a surgical knife (Fig. 7, I in a). If the site is actually the aboral nerve ring, no GABA response should occur. In the second experiment, since the gonoduct consisted of several kinds of cell, one lobule was removed from the gonoduct with surgical knife (Fig. 7, II in a). In this experiment, the supposed site was found to be the gonoduct. In the third experiment, since the gonad was in contact with the peritoneum, one gonad was separated from the surroundings by cutting the peritoneum and border of the gonoduct (Fig. 7, III in a). The supposed site was the gonad. The RR of three different experiments exhibited no significant difference with that of the control (Fig. 7b), indicat-

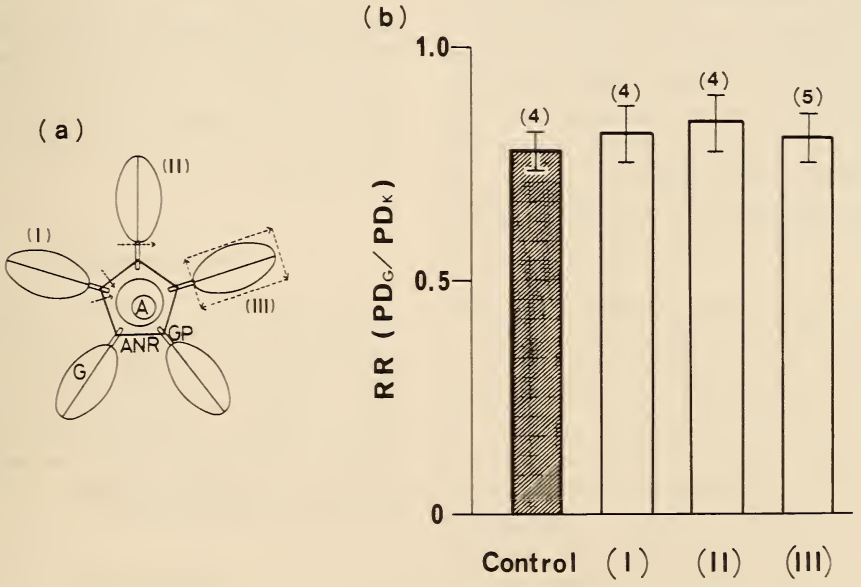

FIG. 7. Site(s) of action of injected GABA. (a) Three different experiments (I, II and III) were carried out. Arrows indicate surgical knife cut. A, anus; ANR, aboral nerve ring; G, gonad; GP, gonopore. (b) The results obtained by (a) are shown in the columns. The number of preparations examined appears in parentheses. Abscissa, results for control and each experiment (I, II and III); ordinate, relative response (RR).

ing GABA to likely act directly on the gonad. When separating the gonad from the peritoneum and gonoduct (Fig. 7, III in a), small rhythmical contractions but no large ones were noted by gonad.

DISCUSSION

It is well established that starfish spawning is triggered by the release of gonad-stimulating substances (GSS) from supporting cells in nervous tissue containing radial nerves [15]. Recently, Shirai and co-workers [16] found two kinds of peptides with GSS action in *Asterias amurensis*. A water extract of radial nerves in the sea cucumber is capable of inducing the spawning of the same species and is possibly a peptide [17]. In the sea urchin, radial nerve water extract has also been reported to bring about gamete shedding [9, 10]. Iwata and Fukase [8] found that acetylcholine, as a neuro-transmitter, induces sea urchin spawning. Recently, gamete shedding in the sea urchin has been observed to occur by injecting GABA [11]. The inhibitory substances depressing spawning activity are known to be present in coelomic fluid [18]. The sea urchin thus begins releasing gametes by washing ceolomic fluid with natural seawater. These substances have been identified as L-glutamic acid and aspartic acid [19], possible neuro-transmitters. Thus, sea urchin spawning appears to be associated particularly with a certain substance(s) from nervous tissue.

The final step in gamete shedding is a contraction of smooth muscle in the gonad wall. Since a ripe gonad in the sea urchin is easily injured in the course of an operation, the extent of gonadal contraction can be determined indirectly by measuring PD. Along with its development and maturation, the mechanical response of a gonad to GABA increases. Moreover, maturing and ripe ovaries display both phasic and rhythmical contraction while young and growing ovaries, only phasic contraction. GABA response is likely to vary according to sex differences. The time required for mechanical response due to GABA in maturing sea urchins is about 20 min. On the dose-response curve, the threshold, 50% effective, and maximal concentrations are 0.01 mM, 0.1 mM and 10 mM, respectively. Our surgical experimental data indicated that GABA acted directly on the gonad and induced contraction and spawning. Thus, the presence of GABA-responding nerve cells in the gonad appears to induce sea urchin spawning.

Recently, it has been reported that the five lobules of sea urchin gonads contract synchronously and rhythmically through the aboral nerve system [13]. Okada *et al.* [13] suggested that the gonoduct generated this rhythm. From the data of the present study, it is evident that a lobule separated from the surroundings is also capable of inducing small but not large rhythm. This in turn appears to result in contraction of the gonad itself and vibration of the rhythmical contraction of the other four lobules. The generator of this rhythm should be studied cytologically.

In conclusion, gonad response to GABA appears to be both phasic and rhythmical contractions, each possibly originating from a different generator. That is, phasic contraction was noted to occur throughout the year, and rhythmical contraction, at the time of gonad maturation. Gonad contraction and spawning appear to be explained quite well if at least two different generators are assumed to be operative. At any rate, GABA appears to act on both these different generators.

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REFERENCES

1 Palmer, L. (1937) The shedding reaction in *Arbacia punctulata*. Physiol. Zoöl., **10**: 352–367.

2 Harvey, E. B. (1939) A method of determining the sex of *Arbacia*, and a new method of producing twins, triplets and quadruplets. Biol. Bull., **77**: 312.

3 Harvey, E. B. (1940) A note on determining the sex of *Arbacia punctulata*. Biol. Bull., **79**: 363.

4 Iwata, K. S. (1950) A method of determining the sex of sea urchins and of obtaining eggs by electric stimulation. Annot. Zool. Japon., **23**: 39–42.

5 Harvey, E. B. (1952) Electrical method of "sexing" *Arbacia* and obtaining small quantities of eggs. Biol. Bull., **102**: 284.

6 Harvey, E. B. (1953) A simplified electrical method of determining the sex of sea urchins and other marine animals. Biol. Bull., **105**: 365.

7 Iwata, K. S. (1962) A simplified electrical method of determining the sex of sea urchins. Zool. Mag., **71**: 301–302.

8 Iwata, K. S. and Fukase, H. (1964) Artificial spawning in sea urchins by acetylcholine. Biol. J. Okayama Univ., **10**: 51–56.

9 Cochran, R. C. and Engelmann, F. (1972) Echinoid spawning induced by a radial nerve factor. Science, **178**: 423–424.

10 Cochran, R. C. and Engelmann, F. (1976) Characterization of spawning-inducing factors in the sea-urchin, *Strongylocentrotus purpuratus*. Gen. Comp. Endocrinol., **30**: 189–197.

11 Takahashi, N. (1986) The spawing of the sea-urchin, *Strongylocentrotus intermedius*, by γ-aminobutyric acid. Bull. Japan. Soc. Sci. Fish., **52**: 2041.

12 Takahashi, N. (1986) Gamete shedding by sea urchins in response to γ-aminobutyric acid. Dev. Growth Differ., **28**: 384.

13 Okada, Y., Iwata, K. S. and Yanagihara, M. (1984) Synchronized rhythmic contractions among five gonadal lobes in the shedding sea urchins: coordinative function of the aboral nerve ring. Biol. Bull., **166**: 228–236.

14 Kawaguchi, S. (1965) Electron microscopy on the ovarian wall of the echinoid with special references to its muscles and nerve plexus. Biol. J. Okayama Univ., **11**: 66–74.

15 de Angelis, E., Viglia, A. Watanabe, T., Shirai, H., Kubota, J. and Kanatani, H. (1972) Presence of granules containing gonad-stimulating substance in starfish radial nerve. Annot. Zool. Japon., **45**: 16–21.

16 Shirai, H., Bulet, P., Kanatani, H., Kondo, N., Imai, K., Isobe, M., Goto, T.and Kubota, I. (1985) Purification of gonad-stimulating substance in starfish. Zool. Sci., **2**: 912.

17 Maruyama, Y. K. (1985) Holothurian oocyte maturation induced by radial nerve. Biol. Bull., **168**: 249–262.

18 Okada, Y. and Iwata, K. S. (1985) A substance inhibiting rhythmic contraction of the gonad in the shedding sea urchin. Zool. Sci., **2**: 805–808.

19 Nogi, H. and Yoshida, M. (1984) Inhibitory substance of rhythmic contraction in sea urchin gonad. Zool. Sci., **1**: 873.